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The Adsorption of Prothrombin to Phospholipid Monolayers Quantitated by Ellipsometry*

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We investigated by means of an automated ellipsometer the calcium-dependent binding of prothrombin from a buffer solution to monolayers of dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidylcholine (DOPC) deposited on chromium slides. This technique allows direct measurements of bound and free protein concentrations and is not hampered by calcium-induced aggregation of vesicles. For pure DOPS a dominant class of binding sites exists with a dissociation constant, $K_d = (6 \pm 2) \times 10^{-10}$ M (mean ± S.D.) and maximal binding of prothrombin, $\Gamma_{max} = 0.26 \pm 0.03$ $\mu g/cm^2$. Incorporation of a small fraction of DOPC in the monolayer causes a large decrease in the binding affinity with a pronounced biphasic behavior of the binding curve. For monolayers consisting of 20% DOPS and 80% DOPC the binding curve becomes monophasic with $K_d = (1.6 \pm 0.6) \times 10^{-7}$ M and $\Gamma_{max} = 0.22 \pm 0.03$ $\mu g/cm^2$.

The procoagulant activity of the monolayers was tested by measuring the generation of thrombin after addition of prothrombin and activated coagulation factors X and V. The thrombin-generating capacity of monolayers and single-bilayer vesicles is comparable but is apparently diffusion limited in the monolayer system.

The calcium-dependent formation of stacked multilayers according to the Blodgett technique appeared to be strongly influenced by the DOPS/DOPC ratio in the phospholipid monolayer.

From these results it is concluded that for pure DOPS monolayers high-affinity prothrombin-phospholipid and phospholipid-phospholipid interactions exist which are radically disturbed when the monolayer contains more than 20–30% of DOPC.

Various phospholipid membranes have served as models for the study of protein-phospholipid interactions in blood coagulation. For the binding of prothrombin to single-bilayer vesicles prepared from PS[1]-PC mixtures, roughly identical binding parameters were found with a variety of techniques based on fluorescence, light scattering, and gel filtration (1–3). Much higher binding constants were found for the binding of prothrombin to monolayers of pure PS spread on a Langmuir film balance (4) and to pure PS multilayers stacked on chromium slides (5). In all these studies the binding process was dependent on the presence of calcium.

A parallel situation is observed in studies on the fusion of biological membranes. In studies on phospholipid vesicles, mixtures of PS and PC require much higher calcium concentrations to induce membrane fusion and have lower calcium-phospholipid binding constants than pure PS (6–8).

The presence study is focused on the effect of phospholipid composition on the binding affinity for prothrombin. The recently introduced ellipsometric technique with phospholipid membranes deposited on chromium slides (5) is especially suited for this problem, because studies on vesicle suspensions at physiological calcium concentrations are hampered by vesicle aggregation for high PS content of the vesicle membranes. Results obtained from prothrombin-phospholipid binding experiments are compared with phospholipid-phospholipid interactions as measured with the Blodgett-Langmuir multilayer stacking technique and with differences in thrombin-generating capacity of the phospholipid membranes.

MATERIALS AND METHODS

Preparation and Characterization of Monolayers—DOPC was purchased from Sigma, and DOPS was prepared from DOPC by enzymatic conversion according to Comfurius and Zwaal (9). Mixtures of DOPS and DOPC in chloroform (2 mg/ml) were spread on the aqueous subphase of a preparative Langmuir trough (Lauda, type FW-1), and the resulting monomolecular film was kept at a constant surface pressure of 40 dynes/cm. The subphase consisted of filtered deionized water (Millipore) with 50 μM EDTA and was kept at 18 °C. A chromium-coated glass slide was dipped into the trough in order to deposit a phospholipid film on the slide. Further details of this technique have been published (5).

As the DOPS/DOPC ratio in the film deposited on the slide could differ from the DOPS/DOPC ratio in the phospholipid film on the trough because of a possible phase separation during the slow depositing process, the DOPS content of the film was checked with ^{14}C-labeled DOPS (Amersham Corp.; specific activity, 60 mCi/mmol). The mass of phospholipid deposited on the slide was determined by ellipsometry, and the DOPS content was calculated by measuring the radioactivity (Packard Tri-Carb counter model 3385) removed from the slide in diethyl ether (Merck). Phospholipid concentrations were determined by phosphate analysis according to the method of Böttcher *et al.* (10). No significant differences were observed between the DOPS/DOPC ratios in the films on the trough and on the slides in the range from 15 to 100% DOPS.

Stacking of Multilayers—Addition of extra phospholipid bilayers by repeated dipping of the slide into the Langmuir trough was measured as a function of the calcium concentration in the subphase and the DOPS/DOPC ratio. The stacked mass was measured in air by ellipsometry, and stacking was considered disturbed if less than 90% of the normal value of 0.40 $\mu g/cm^2$/bilayer was deposited on the slide.

Preparation of Vesicle Suspensions—Bilayer vesicle suspensions were prepared by sonication of a 20% DOPS/80% DOPC mixture for 10 min at 0 °C with an MSE Mark-II ultrasonicator set at 7 μm amplitude as described by De Kruijff *et al.* (11). The concentration of

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[1] The abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine.

phospholipid was 0.3 mM in 0.05 M Tris buffer (pH 7.5) containing 0.1 M NaCl. Unless stated otherwise this buffer was used throughout the study.

Ellipsometry—The slide is placed in a quartz cuvette filled with buffer, and monochromatic light (Spectra-Physics laser model 120, λ = 632.8 nm) is reflected against the slide at an angle of incidence of 68. Before the cuvette is reached, the light passes through a polarizing prism (P) and a quarter wavelength plate. After reflection the light passes through a second polarizing prism (A) and is detected by a photodiode. The prisms P and A are automatically rotated such that the light intensity reaching the photodiode is kept minimal. The optical constants of the reflecting chromium surface can be determined from the initial positions of P and A. Knowing these constants, the refractive index n and the thickness d of a phospholipid or a protein film deposited on the slide can be calculated from the corresponding changes in the positions of P and A. The mass of a substance deposited on the slide can be calculated from the values of n and d by using a modified Lorentz-Lorenz relation,

$$\Gamma = 3d(n^2 - n_b^2)/[(n^2 + 2)\ (r(n_b^2 + 2) - v\ (n_b^2 - 1))] \quad (1)$$

where r and v are, respectively, the specific refractivity and the partial specific volume of the substance deposited on the slide, both expressed in ml/g, and n_b is the refractive index of the buffer. If d is expressed in cm, Γ is obtained in g/cm^2. Experimental values of Γ are more conveniently expressed in μg/cm^2 and, therefore, d is expressed in 10^{-6} cm or 10^{-8} m (5).

Equation 1 is valid if the substance deposited on the slide is mixed with buffer and the value of n remains below the refractive index of the pure substance. If n approaches the latter value, Equation 1 must be replaced by the equation for a pure substance.

$$\Gamma = d(n^2 - 1)/[r(n^2 + 2)] \quad (2)$$

Detailed descriptions of the ellipsometer, the analysis of data, and the validation of Equations 1 and 2 have been published (5, 12).

Specific Refractivities—The specific refractivity r is an inherent property of the substance considered and is not dependent on temperature or concentration. It can be calculated from the molecular structure of the substance involved (13). A value of r = 0.274 ml/g was used for DOPS and DOPC (*cf.* Ref. 5 where the notation A/M was used for r). For prothrombin a value of r = 0.236 ml/g was estimated from the amino acid composition of this protein (5), but this value may contain some error because of unknown bonding involved in the secondary and tertiary structure of the prothrombin molecule. A slightly different value for r is obtained from experimental data on the refractive index increment, dn/dc, of prothrombin solutions. The protein concentration in the film deposited on the slide as calculated from Equation 1 is $c = \Gamma/d$ g/ml. Using this relation one obtains after differentiation of Equation 1 with respect to c in the limit $c \rightarrow 0$,

$$r = 6n_b(dn/dc)/(n_b^2 + 2)^2 + v(n_b^2 - 1)/(n_b^2 + 2). \quad (3)$$

Substituting the values n_b = 1.334, as determined by refractometry for the buffer at 37 °C, v = 0.710 ml/g (14), and dn/dc = 0.192 ml/g (2) into Equation 3, a value of r = 0.254 ml/g is obtained for prothrombin, and this value was used in the present study. This 8% increase in the value of r does not invalidate the verification of Equation 1 for radiolabeled prothrombin as reported before (5). In fact, the corresponding small reduction in values of Γ measured by ellipsometry slightly improves the agreement with direct estimates of Γ from adsorbed radioactivity (*cf.* Ref. 5).

Partial Specific Volumes—Several authors have determined the partial specific volume of phosphatidylcholine in buffer containing 0.1 M NaCl in the temperature range from 20 to 45 °C (15–17). Values for egg PC, dimyristoyl PC, and dipalmitoyl PC obtained in these studies range from 0.973–0.989 ml/g. An average value for DOPC of v = 0.980 ml/g was used in the present study. The partial specific volume v of DOPS was determined by measurement of six stacked double layers of DOPS in buffer containing 10 mM $CaCl_2$. The value of Γ in these experiments was obtained from the monolayer surface area disappearing from the trough during stacking. Knowing Γ, Equation 1 was used to calculate v, and a value of v = 0.890 ± 0.013 ml/g (mean ± S.D.) was obtained at 37 °C. This value is in close agreement with the value of v = 0.889 ml/g obtained for stacked multilayers in air (5).

Measurement of the Procoagulant Activity of Phospholipids—Vesicle suspensions or monolayers deposited on slides were incubated at 20 °C in 4 ml of well-stirred buffer containing 5 mM $CaCl_2$ and 0.5 mg/ml ovalbumin. Various concentrations of factor Xa and a large excess of factor Va were added, and the mixture was incubated for 1 h in order to allow complete equilibration of the adsorption of factors Xa and Va to the phospholipid monolayer. In the absence of phospholipids, no loss of factor Va or Xa was observed in the incubation period. The reaction was started by adding prothrombin. Thereafter serial subsamples were taken and added to plastic cuvettes filled with buffer containing 10 mM EDTA, 0.5 mg/ml ovalbumin, and the chromogenic substrate for thrombin S-2238 (AB-Kabi Diagnostica). The thrombin concentration in the subsamples was determined from the rate of change in adsorbance at λ = 405 nm as recorded on an Aminco DW-2 spectrophotometer. Details of this procedure and the preparation of bovine coagulation factors Va and Xa have been published (18–20). Thrombin generation was negligable in the absence of phospholipid.

Measurements of Prothrombin Binding—Experiments were performed in a cuvette filled with 4 ml of buffer containing 10 mM $CaCl_2$. The buffer was continuously stirred and kept at a temperature of 37 ± 0.2 °C by means of a Peltier element. Bovine prothrombin, prepared according to the method of Owen *et al.* (21), was also incubated in buffer containing 10 mM $Cacl_2$. After adding the prothrombin to the cuvette the adsorption to the slide was followed by measuring the new positions of P and A every 3–5 s. Equilibrium was attained after 10–60 min, depending on the prothrombin concentration; thereafter a subsequent dose of prothrombin was added. Calcium titrations in the presence of excess prothrombin were also performed in this way.

For the lowest prothrombin concentrations used, the adsorption to the slide causes significant depletion of prothrombin from the buffer. Therefore, the adsorptions on pure DOPS for a buffer prothrombin concentration of c = 0.1 μg/ml (*cf.* Fig. 2) were measured by continuous flow of 500 ml of the prothrombin buffer solution through the cuvette during 4 h.

Binding data were analyzed from double-reciprocal plots according to the following equation.

$$1/\Gamma = (K_d/\Gamma_{max})(1/c) + 1/\Gamma_{max}$$

The dissociation constant K_d and the maximal binding capacity Γ_{max} were calculated from, respectively, the horizontal and vertical intercepts.

RESULTS

Binding of Prothrombin as a Function of the DOPS/DOPC Ratio—Fig. 1 shows a typical adsorption experiment. These data allow accurate determination of the adsorbed mass Γ in spite of the fluctuations in the values of n and d (5). Prothrombin adsorption is more than 95% reversible by addition of excess EDTA, and no detectable adsorption is observed in the absence of $CaCl_2$ or on pure DOPC monolayers. Figs. 2 and 3 show binding curves and double-reciprocal plots obtained from the equilibrium values of Γ as a function of prothrombin concentrations c. The biphasic behavior observed for monolayers containing high percentages of DOPS disappears at DOPS percentages below 20%. As shown in Table I, binding sites with a value of K_d of approximately 10^{-7} M are found in all cases, but an additional class of binding sites with much higher binding affinity appears at the higher DOPS contents. Mean values of 5 experiments on 20% DOPS were K_d = (1.6 ± 0.6) × 10^{-7} M and Γ_{max} = 0.22 ± 0.04 μg/cm^2 (mean ± S.D.). Mean values for the high-affinity sites on 100% DOPS were K_d = (5.9 ± 1.8) × 10^{-10} M and Γ_{max} = 0.26 ± 0.03 μg/cm^2. Table I also contains some data from the literature to be discussed hereafter. In this table, the surface area of vesicles was estimated by assuming an area per phospholipid molecule of 0.6 nm^2 and a 2:1 ratio for the distribution of phospholipids between outer and inner monolayers (11).

The Effect of Calcium on Prothrombin Binding—Fig. 4 shows the effect of calcium on the binding of prothrombin at a concentration of 100 μg/ml. Equilibrium values of Γ are shown in this figure as reached after stepwise addition of $CaCl_2$ in 10–60 min. The half-saturation Ca^{2+} concentrations are 0.28 μM for pure DOPS and 1.2 mM for 20% DOPS.

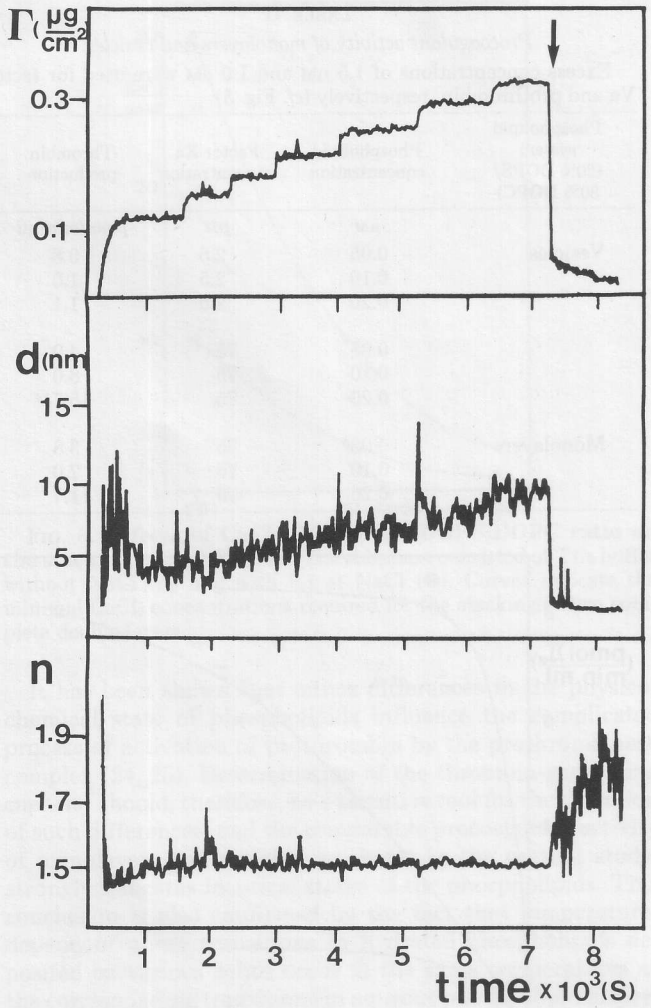

FIG. 1. **Thickness *d*, refractive index *n*, and adsorbed mass Γ during stepwise adsorption of prothrombin to a 80% DOPS/20% DOPC monolayer.** Addition of EDTA is indicated.

Procoagulant Activity of DOPS/DOPC Monolayers and Vesicles—Comparison of the thrombin-generating capacity of vesicles and monolayers requires experimental conditions in which the quantity of lipid is rate limiting in the production of thrombin. Table II shows that, in the presence of excess factor Va and excess prothrombin, a factor Xa concentration of 75 pM will indeed result in a linear dependence of thrombin production on the lipid concentration in the range of 0.05–0.20 μM phospholipid. This latter range was chosen because it could be simply covered experimentally by incremental dipping of the phospholipid-covered slide into the reaction mixture. Fig. 5 shows that the thrombin-generating capacity of both systems is indeed comparable for sufficiently high concentrations of prothrombin. Lower prothrombin concentrations affect the production of thrombin much earlier for monolayers than for vesicles. This suggests that the monolayer system becomes diffusion limited for prothrombin concentrations lower than about 70 μg/ml. The steady-state rate of thrombin production cannot exceed the rate of diffusion of prothrombin from the buffer to the slide, which is dependent on the prothrombin concentration in the buffer. Fig. 5 also shows that a 100% DOPS monolayer has a much lower procoagulant activity than a 20% DOPS monolayer. Verification of this phenomenon for vesicle suspensions is not possible because of vesicle aggregation at DOPS concentrations exceeding 40%.

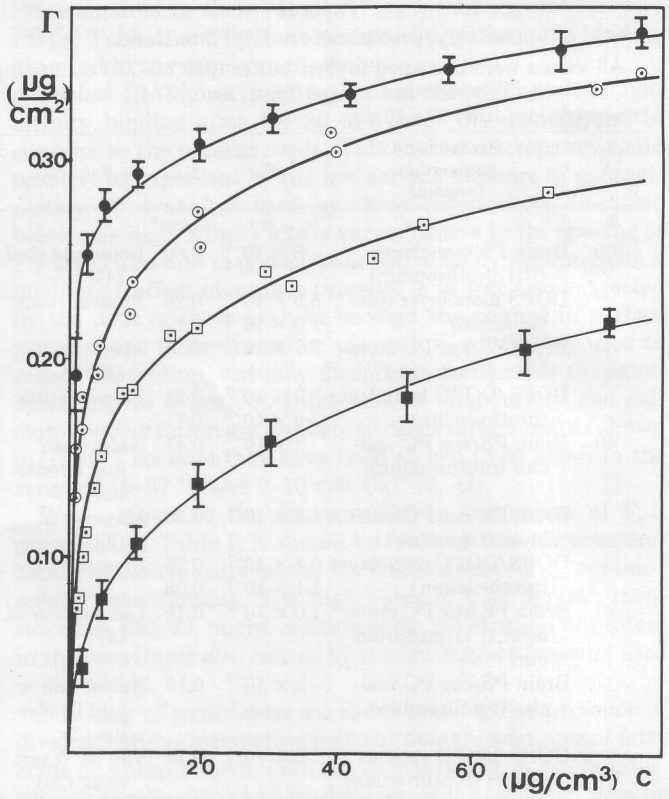

FIG. 2. **Equilibrium values of the adsorbed mass of prothrombin Γ as a function of the free prothrombin concentration *c*.** *Bars* indicate standard error of the mean (n = 5). The phospholipid composition of the DOPS/DOPC monolayers is indicated: 20% DOPS (■), 40% DOPS (⊡), 80% DOPS (⊙) and 100% DOPS (●).

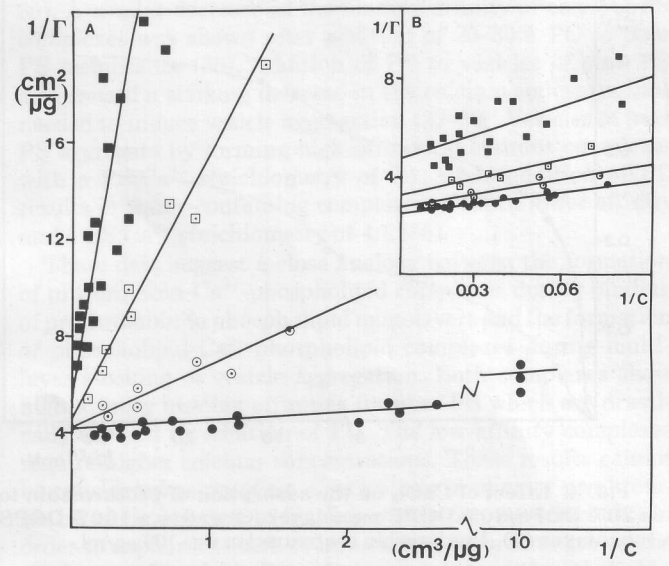

FIG. 3. **Double-reciprocal plots of the variables shown in Fig. 2.** High- and low-affinity values of K_d and Γ_{max} were calculated from, respectively, plot *A* and plot *B*.

Effects of $CaCl_2$, NaCl, and DOPS/DOPC Ratio on Stacking of Multilayers—Fig. 6 shows the minimal $CaCl_2$ concentrations required in the subphase for the stacking of two complete double layers, as a function of the DOPC content of the monolayer on the trough. Results are shown in the absence of NaCl and for 0.1 M NaCl in the subphase. A striking increase in the calcium requirement for increasing DOPC

TABLE I

Binding of prothrombin to PS/PC mixtures

All values were measured in Tris buffer (pH 7.0–7.5) containing 0.1 M NaCl. Temperatures ranged from 10 to 37 °C and calcium concentrations from 2 to 10 mM.

PS content	Phospholipid preparation (protein)	K_d	Γ_{max}	Reference
%		*M*	$\mu g/cm^2$	
100	Brain PS monolayer ([^{3}H]prothrombin)	8×10^{-9}	0.60	Lecompte *et al.* (4)
	DOPS monolayer (prothrombin)	5.9×10^{-10}	0.26	Present study
		1.0×10^{-7}	0.12	
80	Brain PS/egg PC monolayer (fragment 1)	1.2×10^{-7}	0.35	Mayer *et al.* (23)
	DOPS/DOPC monolayer (prothrombin)	9×10^{-9}	0.24	Present study
		1.2×10^{-7}	0.11	
40	Brain PS/egg PC vesicles (prothrombin)	3×10^{-7}	0.41	Nelsestuen and Broderius (2)
	Brain PS/egg PC monolayer (fragment 1)	1.3×10^{-7}	0.35	Mayer *et al.* (23)
	DOPS/DOPC monolayer (prothrombin)	0.4×10^{-7}	0.23	Present study
		1.1×10^{-7}	0.08	
20	Brain PS/egg PC monolayer ([^{3}H]prothrombin)	1.0×10^{-8}	0.15	Lecompte *et al.* (4)
	Brain PS/egg PC vesicles (prothrombin)	3×10^{-7}	0.15	Nelsestuen and Broderius (2)
	DOPS/DOPC vesicles (large) (prothrombin)	2×10^{-7}	0.14	Van de Waart *et al.* (20)
	Brain PS/egg Pc monolayer (prothrombin)	0.7×10^{-7}	0.96	Mayer *et al.* (23)
	DOPS/DOPC monolayer (prothrombin)	1.6×10^{-7}	0.22	Present study

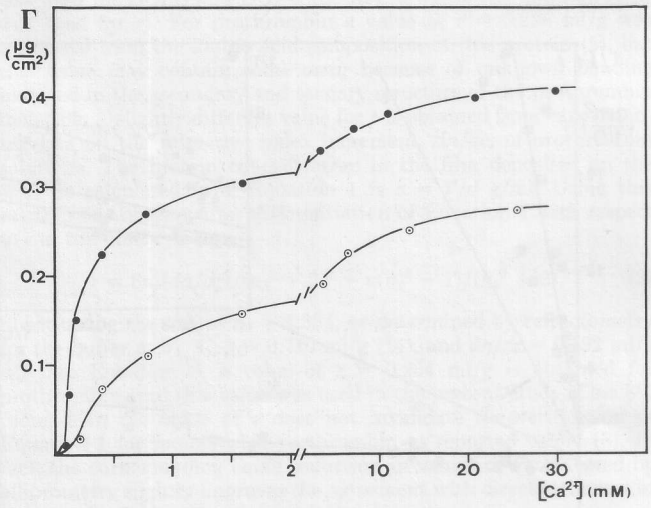

FIG. 4. **Effect of $CaCl_2$ on the adsorption of prothrombin to a 20% DOPS/80% DOPC monolayer (⊙) and to a 100% DOPS monolayer (●).** Prothrombin concentration was 100 μg/ml.

content, or in the presence of NaCl, is apparent. No multilayers can be obtained for a DOPC content exceeding 25%. Even for pure DOPS, multilayer stacks could only be obtained in a narrow range of surface pressures at 38–42 dynes/cm. At higher surface pressures the phospholipid films will collapse, and lower pressures result in incomplete stacking.

DISCUSSION

The refractive index *n* and thickness *d* of a film of organic material deposited on a reflecting surface can be measured by ellipsometry even in the submonolayer range. It was shown, for instance, that correct values of *n* and *d* are obtained for fatty acids spread on a mercury surface for 5–100% of full monolayer coverage (22). As a rule, however, values of *n* and *d* determined for films of less than 10-nm thickness have considerable experimental scatter (*cf.* Fig. 1). This scatter is not random because over- or underestimates of *n* correspond respectively to under- or overestimates of *d*. More specifically, the quantity $d(n - n_b)$ can be determined accurately in spite of fluctuating values of *n* and *d*. This explains why values of Γ, the amount of material per unit surface, as calculated from Equation 1 can be accurate even for layers less than 5-nm thickness (5).

An important issue in the present study is the comparability of phospholipid monolayers deposited on chromium and phospholipid bilayers or biological membranes. As demonstrated under "Materials and Methods," the DOPS/DOPC ratio in the phospholipid mixture is not disturbed by the deposition on chromium. Good miscibility has also been demonstrated for brain PS and egg PC in monolayers at the air/water interface (23). Still, the close proximity of the solid matrix could influence the physical state of the monolayer.

TABLE II

Procoagulant activity of monolayers and vesicles

Excess concentrations of 1.5 nM and 1.0 μM were used for factor Va and prothrombin, respectively (*cf.* Fig. 5).

Phospholipid system (20% DOPS/ 80% DOPC)	Phospholipid concentration	Factor Xa concentration	Thrombin production
	μM	pM	pmol/min/ml
Vesicles	0.05	2.5	0.8
	0.10	2.5	1.0
	0.20	2.5	1.1
	0.05	75	4.0
	0.10	75	8.0
	0.20	75	15.7
Monolayers	0.05	75	3.8
	0.10	75	7.0
	0.20	75	11.7

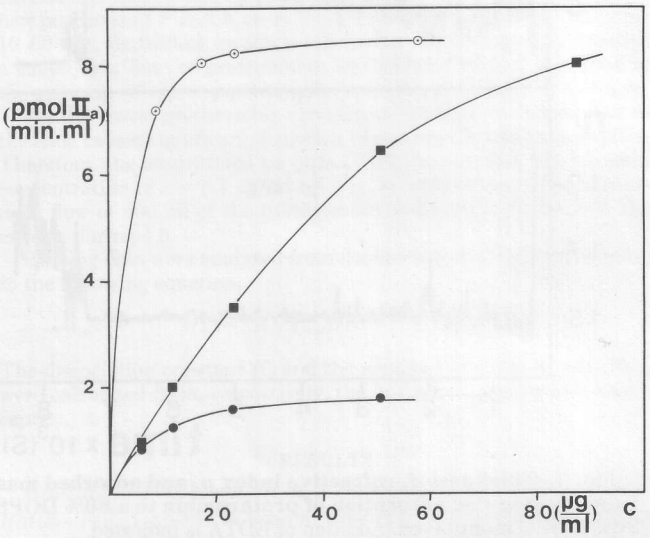

FIG. 5. **Procoagulant activity as a function of the prothrombin concentration *c* for 20% DOPS/80% DOPC vesicles (⊙), 20% DOPS/80% DOPC monolayers (■), and 100% DOPS monolayers (●).** Concentrations of factor Va, Factor Xa, and phospholipid were 1.5 nM, 75 pM, and 0.10 μM, respectively.

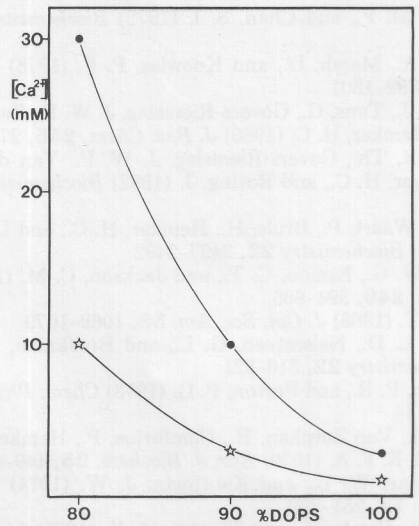

FIG. 6. **Effects of $CaCl_2$, NaCl, and DOPS/DOPC ratio on the stacking of multilayers.** The subphase consisted of Tris buffer without NaCl (☆) and with 0.1 M NaCl (●). Curves indicate the minimal $CaCl_2$ concentrations required for the stacking of two complete double layers.

It has been shown that minor differences in the physicochemical state of phospholipids influence the complicated process of activation of prothrombin by the prothrombinase complex (24, 25). Determination of the thrombin-generating capacity should, therefore, be a sensitive tool for the detection of such differences, and the comparable procoagulant activity of monolayers and vesicles, as shown in the present study, strongly indicates identical states of the phospholipids. This conclusion is also confirmed by the fact that temperature-dependent phase transitions in hydrated phospholipids deposited on various solids occur at the same temperatures as the corresponding transitions in aqueous phospholipid dispersions (26–28). Similar conclusions were reached by comparison of phospholipid monolayer at air/water interfaces with suspensions of liposomes or bilayer vesicles (*cf.* Ref. 29 for a review). With regard to lipid density and phase transitions (30) and also with respect to phospholipid-lipase interactions (31) it was concluded that the physical and biological behavior of both systems is comparable if the surface pressure in the monolayer exceeds 30 dynes/cm.

High-affinity binding on pure PS has also been demonstrated by Lecompte *et al.* (4) (*cf.* Table I). These authors used brain PS, and we have verified that brain PS and pure DOPS monolayers give identical binding curves. A value of K_d of the order of 10^{-10} M could not be measured by these authors because the large surface-to-volume ratio in their system would cause almost complete depletion of protein from the buffer and thus prevents accurate estimation of the free protein concentrations. Similar problems in the measurement of low K_d values exist for light scattering in vesicle suspensions as used by Nelsestuen and Broderius (2). Lecompte *et al.* (4) reported also a value of $K_d = 10^{-8}$ M for a 20% PS monolayer (*cf.* Table I) which contrasts with our results. This discrepancy can probably be explained by the fact that these authors did not use prothrombin concentrations exceeding 5 μg/ml because these tended to solubilize their phospholipid monolayers at the surface pressure of approximately 45 dynes/cm used in their experiments. Determination of the low-affinity binding constant for 20% PS, however, requires measurements in the 10–50% μg/ml range of prothrombin concentrations. This explanation is also suggested by the low value of $\Gamma_{max} = 0.15$ μg/cm² found by these authors for 20% PS, compared to their value of $\Gamma_{max} = 0.60$ μg/cm² for pure PS (*cf.* Table I) and by some nonspecific prothrombin binding observed in the same experiments.

Table I also shows that Mayer *et al.* (23) did not find high-affinity binding sites for 80 and 40% PS monolayers, in contrast to the present study. This second discrepancy could possibly be explained by the low surface pressure of approximately 20 dynes/cm used by these authors. As discussed below, the high-affinity site is very sensitive to the spacing of PS molecules and may disappear below 35 dynes/cm. Such a qualitative effect of surface pressure is in fact also suggested by the data of these authors because the change in surface pressure due to prothrombin adsorption, which they used to record adsorption, virtually disappears for surface pressures exceeding 40 dynes/cm. Influences of temperature and calcium concentration are unlikely to cause differences as shown in Table I because they have been shown to be small in the range of 10–37 °C and 2–10 mM Ca^{2+} (2, 32).

With respect to the discrepancies in the values of Γ_{max} presented in Table I, it should be realized that for measurements in vesicle suspensions the calculation of Γ_{max} contains several uncertainties. The high value of 0.96 μg/cm², mentioned in Ref. 23, might indicate some penetration of protein in the monolayer also caused by the low surface pressure used in this study.

Stacking of multilayers on slides requires the presence of divalent cations interacting with the polar headgroups of fatty acids or phospholipids (33). As shown in Fig. 6, these interactions can be disturbed by displacement of calcium ions by excess sodium or by addition of DOPC to the monolayer. A surface pressure below 38 dynes/cm also prevents successful stacking. Several studies on calcium binding of PS monolayers have demonstrated high binding affinities at high-charge densities, *i.e.* at surface pressures exceeding 30 dynes/cm, and much lower binding affinities at lower surface pressure (34, 35). A similar decrease in the binding affinity of calcium-PS complexes was shown after addition of 20–30% PC to pure PS membranes (36). Addition of PC to vesicles of pure PS also caused a striking increase in the calcium concentrations needed to induce vesicle aggregation (37–39). Vesicles of pure PS aggregate by forming high-affinity anhydrous complexes with a PS:Ca^{2+} stoichiometry of 2:1, while addition of PC results in water-containing complexes of much lower affinity and a PS:Ca^{2+} stoichiometry of 4:1 (36).

These data suggest a close analogy between the formation of prothrombin-Ca^{2+}-phospholipid complexes during binding of prothrombin to phospholipid monolayers and the formation of phospholipid-Ca^{2+}-phospholipid complexes during multilayer stacking or vesicle aggregation. Both complexes show high-affinity binding affinities for pure PS which are drastically lowered by addition of PC. The low-affinity complexes require higher calcium concentrations. These results cannot be considered as proof for electrostatic binding of prothrombin. A chelating type of binding was in fact proposed (32) in order to explain the lack of influence of ionic strength on the calcium-mediated binding of prothrombin to phospholipids. It has also been shown that a Ca^{2+}-dependent transition in the prothrombin molecule is required in order to obtain a molecular form with binding capacity (41). This phenomenon could also be present in high-affinity binding, as shown in Fig. 4 for 100% DOPS, and hampers a comparison with the data on phospholipid-phospholipid interaction shown in Fig. 6.

Normal human plasma prothrombin concentrations range from 8×10^{-7} to 23×10^{-7} M (40) which implies that even the low-affinity binding sites with $K_d = 10^{-7}$ M will be saturated.

Moreover, biological membranes usually have less than 20% of acidic phospholipid while higher percentages are needed for the appearance of high-affinity sites. Both of these factors seem to indicate that these high-affinity sites are physiologically irrelevant.

However, evidence has been presented that clustering of PS molecules may be induced by protein binding to phospholipid membranes (42). If the *in vivo* production of thrombin is a diffusion-controlled process, large differences between the plasma prothrombin concentration and the prothrombin concentration directly adjacent to the procoagulant surface may occur. Under these circumstances it is tempting to speculate that the high-affinity prothrombin-PS complexes may not be devoid of physiological significance.

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